Wahlsten, Jennifer L.

From: Maciej Czerwinski [mczerwinski@xenotechllc.com]
Sent: Thursday, September 18, 2008 10:51 AM
To: Wahlsten, Jennifer L.
Subject: XenoTechniques

Dear Jennifer,

Information contained in the XenoTechniques Vol. 1, No. 1 was disseminated to the public during 12 - 16 October 2003 ISSX Meeting in Providence RI. The Figures 3, 4, 6, 7, 8, 9 and 10 published in XenoTechniques were originally presented in our poster at that meeting. Please see the attached pdf. At the same meeting additional information regarding properties of Fa2N-4 cells was presented by scientists from Roche in the scientific poster number 249.

A pdf version of a final version of XenoTechniques Vol 1., No. 1 was created on July 19, 2004 and has subsequently been used to promote the cell line.

Please feel free to contac us again if we could be of further assitance.

With regards,

Maciej Czerwinski, Ph.D.
Principal Scientist - Cell and Molecular Biology
913 227 7113

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16825 West 116th St. | Lenexa KS 66219 | 913.438.7450 | fax 913.227.7100 | www.xenotechllc.com

INDUCTION OF MAJOR CYTOCHROME P450 ENZYMES IN IMMORTALIZED HUMAN HEPATOCYTES

Maciej Czerwinski,* Kevin C. Lyon,* Martin Perry, Paul Toren, David M. Steen,* Kammie R. Settle,* and Andrew Parkinson*
XenoTech LLC, 16825 W. 116th Street, Lenexa, KS 66219, USA

ABSTRACT

Fa2N-4 is an SV40-immortalized human hepatocyte cell line developed by Multicell Technologies (Warwick, RI). Recently, Mills et al. (*Drug Metabolism Reviews* 34, suppl. 1, #248, 2002) demonstrated that multiple cytochrome P450 (CYP) enzyme mRNAs are inducible in these cells. In the current study, we investigated whether induction of CYP 1A2, 2B6, 2C9, 2C19, and 3A4 in Fa2N-4 cells could be assessed based on enzymatic activity. Cells were grown in MFE media (Multicell Technologies), treated with a prototypical enzyme inducer for 72 hours, and then incubated with CYP-specific substrates followed by LC/MS/MS analysis. In three independent experiments, treatment of Fa2N-4 cells with 20 µM rifampin resulted, on average, in a 3.1-fold increase in bupropion hydroxylase (CYP2B6) activity, a 2.5-fold increase in diclofenac 4'-hydroxylase (CYP2C9) activity and a 5.7-fold increase in midazolam 1'-hydroxylase (CYP3A4) activity. Treatment of Fa2N-4 cells with 100 µM omeprazole caused, on average, a 19-fold induction of phenacetin *O*-dealkylase (CYP1A2) activity. The magnitude of induction of these major drug-metabolizing enzymes and the concentration-response relationship in Fa2N-4 cells were comparable to those in primary cultures of human hepatocytes. The magnitude of induction of CYP3A4 in Fa2N-4 cells cultured in 12-, 24- and 96-well plates was comparable to that in 6-well plates, which bodes well for the development of miniaturized, higher throughput assays. In summary, Fa2N-4 cells demonstrated hepatocyte-like responsiveness to CYP enzyme inducers and present a promising alternative to primary cultures of human hepatocytes for evaluating the enzyme-inducing potential of new chemical entities.

INTRODUCTION

The ability of drug candidates to induce CYP enzymes, particularly CYP1A2 and CYP3A4, is commonly evaluated *in vitro* with primary cultures of human hepatocytes. The supply of human livers available for this purpose is increasingly limited and their response to new chemical entities (NCEs) is variable due to numerous environmental and genetic factors. Recently, Multicell Technologies (Warwick, RI) immortalized human hepatocytes by transfecting primary cultures with DNA of the T antigen of simian SV40 virus. The resulting cell line, Fa2N-4, has been cryopreserved and thawed several times and passaged over forty times. The Fa2N-4 cells retain many characteristics of primary hepatocytes, among them inducibility of multiple CYP enzyme mRNAs in response to enzyme inducers such as rifampin and phenobarbital (Mills *et al.*, 2002). We investigated whether induction of CYPs 1A2, 2B6, 2C9, 2C19, and 3A4 in Fa2N-4 cells could be assessed based on enzymatic activity. In addition, we characterized the inducibility of major CYPs in Fa2N-4 utilizing multiple-well formats and a miniaturized, higher throughput induction screening assay.

MATERIALS & METHODS

The Fa2N-4 cells were plated using proprietary plating and maintenance media optimized to enhance the performance of the cells (Multicell Technologies) on plastieware coated with Vitrogen (Cohesion Technologies, Palo Alto, CA), at 37°C, 5% CO_2, 95% humidity. Typically, cultures of Fa2N-4 cells were grown to confluency in 6-well plates, dosed with 100 µM omeprazole or 20 µM rifampin for 72 hours and incubated with CYP-specific substrates for indicated times. Summary of the LC/MS/MS analytical methods is given below.

Analytical Methods

Enzyme	Substrate	Metabolite	Ionization Mode
CYP1A2	Phenacetin	Acetaminophen	APCI+
CYP2B6	Bupropion	Hydroxybupropion	ESI+
CYP2C9	Diclofenac	4'-Hydroxydiclofenac	ESI-
CYP2C19	*S*-mephenytoin	4'-Hydroxymephenytoin	ESI-
CYP3A4	Midazolam	1'-Hydroxy midazolam	ESI+

APCI+ - Atmospheric Pressure Chemical Ionization, ESI - Electro Spray Ionization

RESULTS

1. Activity and inducibility of CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4 in immortalized Fa2N-4 hepatocytes was investigated with CYP isoform-specific substrates added directly to the culture medium and followed by measurement of their metabolites. Accumulation of CYP-specific metabolites was linear over time for CYP1A2, CYP2B6, CYP2C9, and CYP2C19 for up to 6 hours and up to 2 hours for CYP3A4 in vehicle and inducer-dosed cultures (Figure 1). In multiple experiments, treatment of Fa2N-4 cells with 20 µM rifampin resulted, on average, in a 2.5-fold increase in buproprion hydroxylase (CYP2B6) activity, a 2.0-fold increase in diclofenac 4'-hydroxylase (CYP2C9) activity and a 5.1-fold increase in midazolam 1'-hydroxylase (CYP3A4) activity. Treatment of Fa2N-4 cells with 100 µM omeprazole caused, on average, a 20-fold induction of phenacetin *O*-dealkylase (CYP1A2). The magnitude of induction in Fa2N-4 cells was comparable to that of primary cultures of human hepatocytes in 60-mm dishes (Table 1). The induction of CYP2B6, established in three experiments, demonstrated the consistency of the system's performance (Figure 2). The CYP2C19 activity of *S*-mephenytoin hydroxylase was not induced by treatment of the cells with rifampin.
2. The omeprazole concentration response of CYP1A2 in Fa2N-4 cells reached a peak response at 50 µM, while the concentration response curve of CYP3A4 had maximal response at 20 µM rifampin (Figure 3). For both CYP enzymes, immortalized hepatocytes exhibited peak response at the same concentration as primary cultures of hepatocytes (LeCluyse et al., 2000) Time dependence study of induction of CYP1A2 and CYP3A4 indicated maximal response at 72 hours, in which the Fa2N-4 cells mirrored responsiveness of hepatocytes in primary cultures (Figure 4).
3. In order to assess whether the Fa2N-4 cells can be used in miniaturized screening assay we compared inducibility of CYP1A2, CYP2B6, CYP2C9, and CYP3A4 in 6, 12, 24 and 96-well plates. The inducibility of CYP2B6, CYP2C9, and CYP3A4 was the same across different plate formats, while induction of CYP1A2 in 96-well plate format (examined in a single experiment at 1 hour incubation with phenacetin) was lower in comparison to 6-well plate (Figure 5).
4. Induction of CYP3A4 was evaluated, using several compounds in 24-well plate cultures. The system correctly differentiated known pregnane-X receptor agonists from inducers acting through different nuclear receptor pathways such as aryl hydrocarbon receptor (omeprazole, 3-methylcholanthrene) (Figure 6).

Figure 1: CYP induction in Fa2N-4 cells

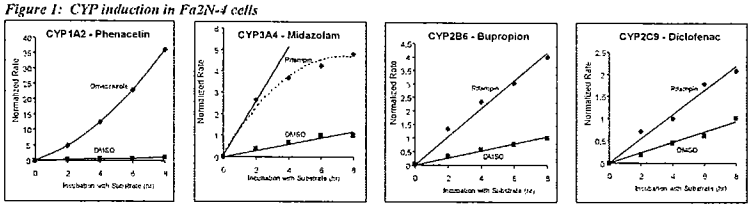

Figure 2: Reproducibility of CYP2B6 induction in Fa2N-4 cells

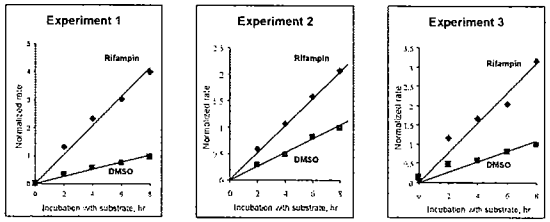

Figure 3: CYP induction in Fa2N-4 cells: inducer concentration response

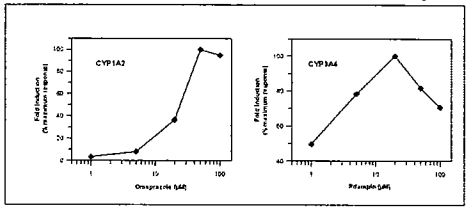

Figure 4: Time course of CYP induction in Fa2N-4 cells

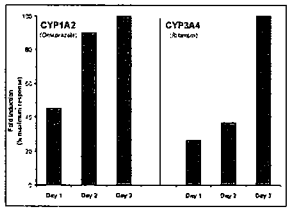

Figure 5: CYP induction in Fa2N-4: multiple plate formats

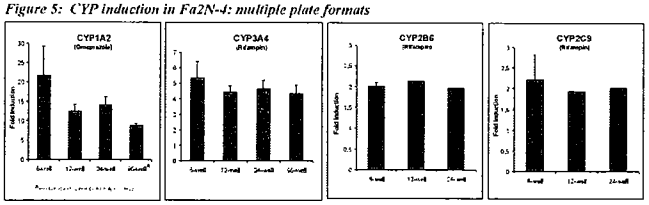

Table 1: CYP induction in vitro in Fa2N-4 cells versus human hepatocytes

Enzyme (Inducer)	Fa2N-4 cells (This report)	Primary cultures (Madan et al., 2003)
CYP1A2 (β-naphthoflavone, omeprazole)	20 fold (9.3–29 fold)	13 fold (2.0–56 fold)
CYP2B6 (Rifampin)	2.5 fold (2.0–3.9 fold)	13 fold (3.0–71 fold)
CYP2C9 (Rifampin)	2.0 fold (1.6–2.8 fold)	3.5 fold (1.5–10 fold)
CYP3A4 (Rifampin)	5.1 fold (4.0–6.9 fold)	10 fold (0.0–145 fold) (Median: 3.8 fold)

CONCLUSIONS

1. The *in vitro* system combining immortalized human hepatocytes and analytical procedures examined in this study provides the ideal solution to screen multiple NCEs for enzyme induction early in the drug development process. The immortalized hepatocytes retain the essential characteristics of cells in primary cultures, such as CYP activity and inducibility, and offer significant advantages over increasingly scarce hepatocytes. The Fa2N-4 cells respond to enzyme inducers in a predictable manner that mimics hepatocytes in magnitude of response, concentration response to prototypical inducers, and the time course of induction. Since the immortalized hepatocytes can be cryopreserved and are readily available, they constitute a reproducible, well-characterized system for induction studies.
2. Robust growth, ease of handling and good responsiveness of Fa2N-4 cells in 12, 24 and 96-well plates bodes well for a rapid development of a higher throughput induction screen.
3. Major nuclear receptor pathways, signaling through AhR and PXR, are functional in Fa2N-4 cells. Therefore, this whole-cell system is superior to gene-reporter assays, which monitor the interaction of the NCE with a single receptor at a time and do not address issue of cross-talk between nuclear receptor pathways.

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Mills JB, Faris R, Cascio S, Liu J and de Morais SM (2002) An HTS assay for induction of enzymes and transporters using a human hepatocyte clonal line and RNA detection *Drug Metabolism Reviews* 34 suppl. 1, #248

2003

Figure 6: Evaluation of effect of known enzyme inducers on CYP3A4 activity in Fa2N-4 cells